J. Clin. Chem. Clin. Biochem.
Vol. 27, 1989, pp. 567–575

Hydrogen Peroxide and Methyl Mercury are Primary Stimuli of Eicosanoid Release in Human Platelets

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(Received April 21/July 20, 1989)

Summary: Hydrogen peroxide (H_2O_2) and methyl mercury induced the liberation of arachidonate and its metabolites from human washed platelets. [^{14}C]Eicosanoids were extracted from the supernatants of [^{14}C]arachidonate-prelabelled platelets and analysed by thin layer chromatography and radioscanning. Thromboxane B_2 (TXB_2), 12(S)-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) were found as stable metabolites, together with unreacted arachidonate. In the presence of dazoxiben, a shift in eicosanoid metabolism was observed towards prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2) and prostglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), while in the presence of indomethacin there was a shift towards 12-HETE and unmetabolized arachidonate. The concentration pattern of those metabolites resembled that found with the physiological agonist, thrombin. H_2O_2 and methyl mercury also induced platelet shape change, aggregation and secretion. The EC_{50} values for the induction of shape change and aggregation were 27 and 850 µmol/l for H_2O_2 and 0.33 and 2.7 µmol/l for methyl mercury, respectively. The [^{3}H]serotonin release required higher stimulus concentrations and amounted to 45% with 2 µmol/l H_2O_2 and to 16% with 3 µmol/l methyl mercury. These effects on platelet function were absent in platelets exposed to acetylsalicylic acid and prevented by indomethacin, the prostaglandin H_2 (PGH_2)/thromboxane A_2 (TXA_2) receptor antagonist, daltroban, and the functional antagonist, iloprost. In contrast, none of these drugs suppressed the formation of [^{14}C]eicosanoids, indicating that the platelet activation by H_2O_2 and methyl mercury essentially requires previous PGH_2/TXA_2 formation. As expected, the thromboxane synthase inhibitor, dazoxiben, did not prevent, but instead potentiated the activation by H_2O_2 and methyl mercury through accumulated PGH_2. These results show that H_2O_2 and methyl mercury selectively trigger the mobilization of endogenous arachidonate. In platelets exposed to acetylsalicylic acid or in the presence of a PGH_2/TXA_2 receptor antagonist, they are model stimuli for investigating endogenous eicosanoid formation without the interference of a receptor-operated activation cascade. Therefore, H_2O_2 and methyl mercury are valuable tools for elucidating the unresolved trigger mechanisms of eicosanoid release and for studying approaches to its selective inhibition.

Introduction

The synthesis of eicosanoids in human platelets is triggered by their physiological agonists thrombin, collagen, ADP etc., but it is not essential for platelet activation by these stimuli (1). Eicosanoid release follows the stimulation of phosphatidylinositol turnover and the rise in the intracellular diacylglycerol and Ca^{2+} concentrations in stimulated platelets (2). PGH_2[1]) and TXA_2[1]) are major but unstable eicosanoids formed from endogenous arachidonate, and they play a functional role as strong feedback agonists of platelet activation (3–5). Acetylsalicylic acid (aspirin®) and other non-steroidal anti-inflammatory drugs reduce platelet activation, because they inhibit

[1]) Abbreviations:
12-HETE, 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid;
HHT, 12(S)-hydroxy-5,8,10-heptadecatrienoic acid;
12-HPETE, 12(S)-hydroperoxy-5,8,10,14-eicosatetraenoic acid;
PG, prostaglandin;
TX, thromboxane;
EC_{50}, concentration of an agonist that produces 50% effect;
TLC, thin layer chromatography.

PGH_2 and TXA_2 formation from endogenous arachidonate released upon platelet stimulation by either of the above mentioned agonists. Antagonists acting on the PGH_2/TXA_2 receptor exert a similar inhibitory effect by preventing PGH_2 and TXA_2 from stimulating their common PGH_2/TXA_2 receptor (6). However, neither cyclooxygenase inhibitors nor TXA_2/PGH_2 receptor antagonists suppress the mobilization of arachidonate, which is the key event in eicosanoid formation (7). Thus, these drugs cannot prevent the mobilization and metabolism of free arachidonate to biologically active products. In particular, cyclooxygenase inhibitors like acetylsalicylic acid may enhance the formation of lipoxygenase products, such as 12-HETE[1]) in platelets (8) and leukotrienes from platelet-derived arachidonate in leukocytes (9). Therefore, there is considerable pharmacological interest in the search for inhibitors of the initiation of eicosanoid release, namely the mobilization of arachidonate.

The biochemical link between the receptor-operated activation cascade and eicosanoid release is still unknown (10, 11), as are specific inhibitors of the arachidonate release (12). Several enzymes regulate the free arachidonate concentration. Phospholipase A_2[2]) supplies the major portion of free arachidonate by liberation from phospatidylcholine and to a lesser extent from phosphatidylethanolamine (13, 14). The sequential action of phospholipase C[2]) and diglyceride and monoglyceride lipases contributes to less than 15 percent of the arachidonate mobilized during platelet stimulation with thrombin (15). On the other hand, arachidonyl-CoA synthetase[2]) (16) and lysophospholipid acyltransferase[2]) (17) can rapidly sequester a large amount of free arachidonate and reincorporate it into the phospholipids by the reacylating pathway (18). Therefore, the extent to which an activation of the deacylation or an inhibition of the reacylation pathway contribute to the arachidonate mobilization is the subject of an ongoing debate (19). The biochemical study of arachidonate release is difficult, because both membrane-bound enzymes and membrane-associated substrates are involved. Another problem arises from the lack of specific biochemical and pharmacological tools to interfere with the complex mechanism that controls the concentration of free arachidonate. Hydrogen peroxide (H_2O_2) and methyl mercury chloride may prove to be such tools, suitable for investigating the underlying mechanism. It is shown in the present work that both agents mobilize arachidonate, independently of the activation cascade. Thus they act more directly on eicosanoid release than thrombin or other stimuli, which require the receptor-operated activation cascade.

H_2O_2 belongs to the reactive oxygen species produced by phagocytosing or otherwise stimulated neutrophils. The amount produced by $2.5 \cdot 10^6$ neutrophils reaches 6 nmol per 20 minutes (20, 21). A much less potent source of H_2O_2 is the platelet stimulated with latex particles or opsonized zymosan (22). In studies on the actions of H_2O_2 on platelets *in vitro*, different authors report on the one hand an inhibition of platelet aggregation induced by arachidonate (21, 23) and an inactivation of the platelet cyclooxygenase (24); and on the other hand, an enhancement by H_2O_2 of the effects of a variety of platelet agonists (25) and its role as a cosubstrate in the activation of the platelet cyclooxygenase[2]) (26). Our own work on the role of reactive oxygen species in platelet physiology (27, 28) led us to use H_2O_2 as a model stimulus of platelet activation via thromboxane formation (29). While H_2O_2 represents a physiological agent, methyl mercury is formed by microorganisms from inorganic mercury in industrial waste and accumulates in man at the end of the food chain through its solubility in lipids. It causes toxic effects on brain, liver and kidneys (for review see l.c. (30)) but its mechanism of action has not yet been clarified. Inhibitory effects of methyl mercury were described on adenylate cyclase (31, 32) and on 12-lipoxygenase[2]) (33), leading to a reduced inhibition of thromboxane synthesis by 12(S)-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE). Additionally, the whole blood glutathione peroxidase activity and total blood glutathione is decreased by methyl mercury, whereas the serum levels of TXB_2[1]) and PGE_1[1]) increase (34). *MacFarlane* (23) reported that methyl mercury induces platelet shape change, aggregation and the release reaction, and that these effects could be suppressed by an inhibition of the cyclooxygenase. The present study demonstrates that the target of H_2O_2 and methyl mercury is the mobilization of endogenous arachidonate. Thus H_2O_2 and methyl mercury are useful model substances for biochemical and pharmacological research on the initial step of eicosanoid release.

Materials and Methods

Biochemicals

Perhydrol (= hydrogen peroxide) No. 7210 and methyl mercury chloride No. 806100 were purchased from Merck (Darmstadt, F.R.G.), thrombin from Behringwerke (Marburg, F.R.G.),

[2]) Enzymes:
Arachidonyl-CoA synthetase (EC 6.2.1.15)
Prostaglandin synthase (Cyclooxygenase) (EC 1.14.99.1)
Lactate dehydrogenase (EC 1.1.1.27)
Lipoxygenase (1.13.11.12)
Lysophospholipid acyltransferase (EC 2.3.1.23)
Phospholipase A_2 (EC 3.1.1.4)
Phospholipase C (EC 3.1.4.3)
Thromboxane synthase (EC 5.3.99.5)

indomethacin No. I-7378 and human albumin, essentially fatty acid free, No. A-3782 from Sigma GmbH (München, F. R. G.). Dazoxiben (UK 37248) was supplied by Pfizer Central Research (Sandwich, UK), Sulotroban (BM 13.177) by Boehringer Mannheim (Mannheim, F. R. G.), Iloprost (ZK 36374) by Schering AG (Berlin, F. R. G.) and LU 41.453, an inhibitor of the cyclic AMP phosphodiesterase, by Knoll AG (Ludwigshafen, F. R. G.). [1-^{14}C]Arachidonic acid (2.0 TBq/mol) and ^{3}H-labelled $PGF_{2\alpha}$, PGE_2, PGD_2, TXB_2 and 12-HETE as standards for thin layer chromatography were obtained from New England Nuclear (Dreieich, F. R. G.). [^{3}H]Serotonin (5-hydroxy[G-^{3}H]tryptamine creatinine sulphate) was obtained from Amersham Buchler (Braunschweig, F. R. G.).

Preparation of washed platelets

Blood was taken only from healthy volunteers, who all disclaimed taking any drugs during the previous week. Disc shaped washed platelets were prepared from whole blood anticoagulated with 1/7 vol ACD NIH-formula A (8 g/l citric acid, 22 g/l sodium citrate, 24.5 g/l hydrous dextrose) according to our method of washing with acid citrate (35). The platelets were sedimented from platelet-rich plasma by 7 min centrifugation at 330 *g* and resuspended in a solution containing NaCl 120 mmol/l, KCl 5 mmol/l, $CaCl_2$ 2 mmol/l, $MgCl_2$ 1 mmol/l, glucose 5 mmol/l, albumin 2 g/l, apyrase 50 mg/l, sodium phosphate/NaOH 30 mmol/l, pH 6.5. When platelets were treated with acetylsalicylic acid 5 mmol/l, a freshly prepared solution of 20 mmol/l in isotonic TES buffer pH 7.4 was added to the platelet-rich plasma and incubated for 30 min at 22 °C prior to the washing procedure. Where platelet secretion was to be measured, platelet-rich plasma was incubated with 0.08 µmol/l (≈ 60 kBq) [^{3}H]serotonin for 10 min at 22 °C in order to label the secretory dense bodies. This procedure could be performed separately or in combination with acetylsalicylic acid treatment. The washed platelets were finally suspended in a test medium containing NaCl 120 mmol/l, KCl 5 mmol/l, $CaCl_2$ 1 mmol/l, $MgCl_2$ 0.1 mmol/l, glucose 5 mmol/l, albumin 0.5 g/l, apyrase 50 mg/l, sodium phosphate 1 mmol/l, TES/NaOH 30 mmol/l, pH 7.4. The final platelet concentrations were $200 \cdot 10^9$/l for the measurements of platelet shape change, aggregation and [^{3}H]serotonin release and $2 \cdot 10^{12}$/l for the measurement of [^{14}C]eicosanoid metabolism.

Rheooptical measurements of shape change and aggregation

Shape change and aggregation were measured turbidimetrically in a dual channel aggregometer (Labor, Ahrensburg, F. R. G.) at 37 °C by the method established by *Born* (36) with modifications as published earlier (35). Shape change experiments were performed in the presence of EDTA 2 mmol/l at a stirring speed of 400 min^{-1}. In aggregation experiments, 300 mg/l of fibrinogen were added and the stirring speed was 1000 min^{-1}. Preincubation of platelet suspensions with test substances started 5 minutes prior to stimulation. The instrumental arrangement for measurements and the quantification of shape change and aggregation were described previously (35).

Release of [^{3}H]serotonin

The secretion of [^{3}H]serotonin from platelet dense granules was determined as the decrease of the platelet bound radioactivity (35). The reuptake of serotonin was inhibited by the presence of imipramine 2 µmol/l. Aliquots were taken from incubates three minutes after addition of the activating agent and were mixed with 50 µl of an 0.1 mmol/l ice-cold EDTA solution pH 7.4, rapidly cooled to 0 °C and centrifuged for 30 s in an Eppendorf 3200 centrifuge at 0 °C. The radioactivity of 200 µl aliquots of the supernatant was counted in a liquid scintillation counter.

Platelet prelabelling with [^{14}C]arachidonate

For radiochemical assays, the washed platelets were prelabelled with [^{14}C]arachidonate. For this purpose platelets ($200 \cdot 10^9$/l) were resuspended in a buffer containing NaCl 120 mmol/l, KCl 5 mmol/l, $MgCl_2$ 1 mmol/l, $CaCl_2$ 2 mmol/l, human albumin 2 g/l, glucose 5 mmol/l and apyrase 50 mg/l in phosphate-buffer 30 mmol/l, pH 6.5. [^{14}C]Arachidonate was dissolved in the same buffer by sonification for 2 minutes. The platelets were incubated with [^{14}C]arachidonate 3.25 µmol/l for one hour at 37 °C. Arachidonate was added in 10 portions at 6 min intervals in order to avoid platelet activation. Then the platelets were washed twice to remove remaining free arachidonate and finally resuspended in test buffer containing NaCl 120 mmol/l, KCl 5 mmol/l, $MgCl_2$ 0.2 mmol/l, $CaCl_2$ 1.2 mmol/l, human albumin 0.5 g/l (if not stated otherwise), glucose 5 mmol/l, phosphate 6 mmol/l, and apyrase 50 mg/l in 0.1 mol/l TES/HCl, pH 7.4. The final platelet concentration was $2 \cdot 10^{12}$/l.

Radiochemical assays

Samples of platelets (980 µl) prelabelled with [^{14}C]arachidonate were warmed to 37 °C for 5 minutes. Drug solutions (10 µl) were added and after another 5 minutes, platelet stimuli were applied in a volume of 10 µl. The final test volume was 1 ml. In the controls, test medium was added instead of drugs. After the desired time of incubation, the reaction was stopped by acidifying to pH 3 with 10 µl of concentrated formid acid. Samples were centrifuged at 10 000 *g* for 15 minutes and the cell-free supernatants extracted twice with ethyl acetate. The combined organic phases were dried under nitrogen, redissolved in 100 µl of chloroform/methanol (2 + 1, by vol.) and analysed by thin layer chromatography. The overall recovery of the radioactivity in the extracts and the platelet sediments averaged 86 percent of the platelet-bound radioactivity after prelabelling.

Analysis of [^{14}C]eicosanoids by radioTLC[1])

Silica gel TLC-plates (No. 11798, Merck, Darmstadt, F. R. G.) were heated to 110 °C for 15 minutes and subsequently allowed to cool in a desiccator. After application, the plates were developed by a solvent system consisting of chloroform/methanol/acetic acid/water (87+8+4+1, by vol.), which resolved well the comigrating authentic standards of 12-HETE, prostaglandins and thromboxane B_2 (fig. 1). The radioactive spots on the TLC-plate were evaluated by a Berthold LB 2842 TLC-linear analyser (Berthold, Wildbad, F. R. G.) with integration software. After peak integration and background subtraction the percentage of radioactivity was calculated for each compound. The liberation of radiolabelled eicosanoids was determined by liquid scintillation counting of aliquots taken from the test suspension and cell-free supernatant. The extracts of the sediments were free of [^{14}C]eicosanoids.

Release of lactate dehydrogenase

As a parameter of cytoplasmic leakage, lactate dehydrogenase[2]) was measured in an optical test (Monotest, Boehringer Mannheim, Mannheim, F. R. G.). Aliquots were taken from the incubates five minutes after the addition of H_2O_2 or methyl mercury, and the supernatants prepared by centrifugation for 30 s in an Eppendorf 3200 centrifuge at 0 °C. The lactate dehydrogenase in the supernatants was compared with the total lactate dehydrogenase released from control platelets after their lysis by three cycles of freezing and thawing.

Statistics

Data are expressed as mean ± S. E. M. Statistical comparisons were performed using *Student*'s t-test for paired data. The null hypothesis was rejected if $p < 0.05$. The concentration effect

curves were calculated from data points by multiple iterations using a non-linear sigmoidal least squares regression fit algorithm developed by *Marquardt* (37) and improved by *Tabata & Ito* (38). The algorithm is part of the statistics and plotter graphics software package GRAPHPAD from ISI (USA).

Results

Treatment of [^{14}C]arachidonate-prelabelled platelets with hydrogen peroxide (H_2O_2) or methyl mercury led to the release of radiolabelled metabolites and unreacted arachidonate. The major [^{14}C]eicosanoids were TXB_2[1]), HHT[1]) and 12-HETE[1]) which appeared in the same relative amounts and representing the same percentage of the liberated [^{14}C]eicosanoids, irrespective of whether the platelets were stimulated with H_2O_2, methyl mercury or thrombin (fig. 1 and tab. 1). With H_2O_2, a rise in the liberation of [^{14}C]eicosanoids continued up to 30 mmol/l, but beyond 1 mmol/l H_2O_2 the amount of TXB_2 and HHT decreased in favour of unreacted arachidonate and 12-HETE (fig. 2). The optimal concentration of H_2O_2 for the stimulation of TXB_2 formation was approximately 1 mmol/l, which induced the liberation of 4.5 ± 0.4% (n = 5) of the platelet-bound radioactivity (fig. 2). With methyl mercury 20 µmol/l and thrombin 10^3 IU/l, the liberation of [^{14}C]eicosanoids amounted to 5.6 ± 0.3% (n = 3) and 7.2 ± 0.5% (n = 3) of the platelet-bound radioactivity, respectively. Inhibitors of the cyclooxygenase, such as acetylsalicylic acid or indomethacin (tab. 1), suppressed the TXB_2 and HHT formation and increased the formation of the 12-lipoxygenase product, 12-HETE, but did not inhibit the [^{14}C]eicosanoid release (5.6 ± 0.3% vs. 4.9 ± 0.5%, $p > 0.1$ for methyl mercury and 4.4 ± 0.4% vs. 4.1 ± 0.3%, $p > 0.1$ for H_2O_2). The thromboxane synthase[2]) inhibitor, dazoxiben, shifted the [^{14}C]eicosanoid metabolism from TXB_2 and HHT to the stable prostaglandins, PGE_2[1]), PGD_2[1]) and $PGF_{2\alpha}$[1]) (tab. 1), which are spontaneously formed from the labile prostaglandin, endoperoxide PGH_2 (2, 39, 40). Iloprost and LU 41.453, which are functional antagonists in platelets, prevented the thrombin-induced [^{14}C]eicosanoid release, but had no effect on the [^{14}C]eicosanoid formation triggered by methyl mercury (tab. 2).

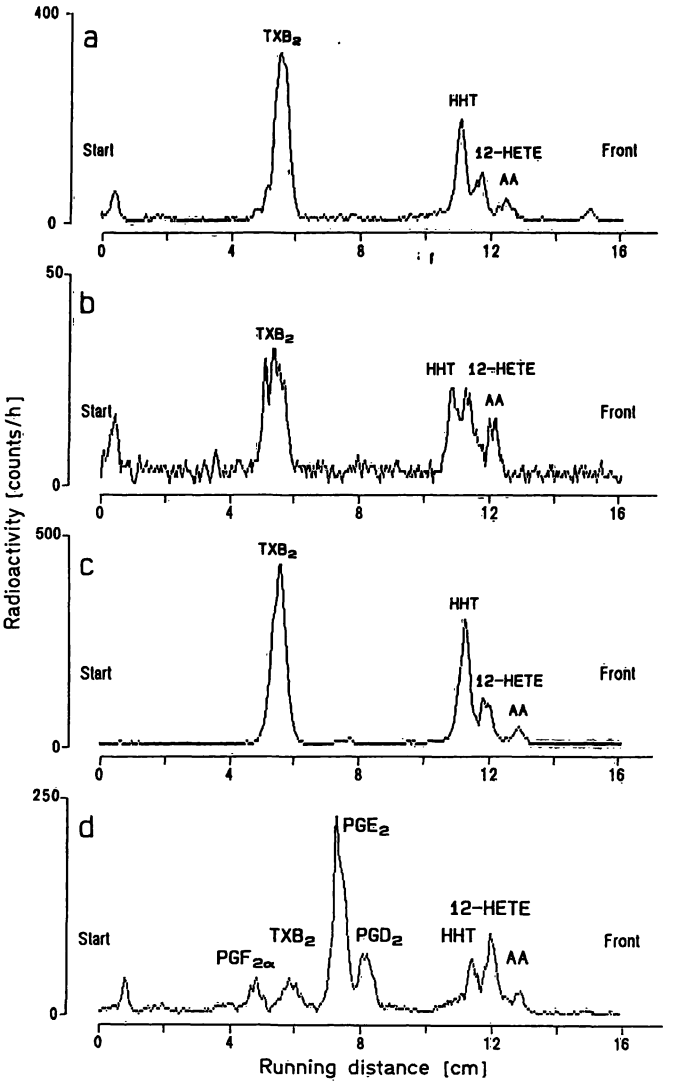

Fig. 1. Thin layer radiochromatograms of [^{14}C]eicosanoids in supernatants from human platelets prelabelled with [^{14}C]arachidonate and stimulated for 1 minute with (a) methyl mercury 20 µmol/l, (b) hydrogen peroxide 1 mmol/l, (c) thrombin 10^3 IU/l and (d) with methyl mercury 20 µmol/l in the presence of dazoxiben 10 µmol/l.

TXB_2 = thromboxane B_2
HHT = 12(S)-hydroxy-5,8,10-heptadecatrienoic acid
12-HETE = 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid
AA = arachidonic acid
$PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$
PGE_2 = prostaglandin E_2
PGD_2 = prostaglandin D_2

H_2O_2 and methyl mercury caused platelet activation as indicated by shape change, aggregation and [^{3}H]serotonin release. The concentration-effect relationships of the shape change and the aggregation are shown on figure 3a and b. The [^{3}H]serotonin release required the highest stimulus concentrations and it amounted to approximately 45% with 2 mmol/l H_2O_2 (fig. 4a) and 16% with 3 µmol/l methyl mercury (fig. 4b) in platelets aggregating in the presence of fibrinogen. If aggregation was prevented by EDTA 2 mmol/l, [^{3}H]serotonin release decreased to 20% and 3% (fig. 4a and 4b), respectively, whereas a full shape change remained unaffected. H_2O_2 was used at concentrations not higher than 2 mmol/l in functional tests, since at higher concentrations it released small bubbles of molecular oxygen that severely interfered with the turbidimetric measurements of shape change and aggregation. The aggregating effect of methyl

Tab. 1. Effects of hydrogen peroxide, methyl mercury and thrombin on the formation of [^{14}C]metabolites in platelets prelabelled with [^{14}C]arachidonate. The platelets were stimulated for 1 minute in the absence (= control) or the presence of dazoxiben, daltroban or indomethacin. Platelet concentration: $200 \cdot 10^9$/l.
12-HETE = 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid
HHT = 12(S)-hydroxy-5,8,10-heptadecatrienoic acid

	[^{14}C]Eicosanoids (percent of total eicosanoids released)							
	Prostaglandin $F_{2\alpha}$	Thromboxane B_2	Prostaglandin E_2	Prostaglandin D_2	HHT	12-HETE	Arachidonic acid	
Hydrogen peroxide 1 mmol/l								
Control	0	31.1 ± 3.3	0	0	18.4 ± 0.6	22.7 ± 2.1	27.9 ± 2.5	(n = 5)
Dazoxiben 50 µmol/l	0.9 ± 0.3	0	22.7 ± 4.6	3.6 ± 1.0	0	36.8 ± 2.5	36.0 ± 4.7	(n = 3)
Indomethacin 50 µmol/l	0	0	0	0	0	66.4 ± 6.8	33.7 ± 4.9	(n = 3)
Methyl mercury 20 µmol/l								
Control	0	38.3 ± 3.5	3.0 ± 1.1	0	22.5 ± 2.1	29.5 ± 2.4	6.8 ± 1.6	(n = 6)
Dazoxiben 50 µmol/l	5.4 ± 2.1	1.4 ± 0.8	42.3 ± 2.5	10.0 ± 1.1	0	34.2 ± 1.6	6.8 ± 1.3	(n = 4)
Indomethacin 50 µmol/l	0	0	0	0	0	61.1 ± 8.4	39.0 ± 5.6	(n = 3)
Daltroban 20 µmol/l	0	40.6 ± 5.6	4.0 ± 1.5	0	28.7 ± 2.3	18.7 ± 5.9	8.0 ± 2.0	(n = 3)
Thrombin 10^3 IU/l								
Control	0	26.4 ± 3.3	1.8 ± 0.4	0	27.5 ± 3.5	22.2 ± 3.0	22.2 ± 3.2	(n = 3)
Dazoxiben 50 µmol/l	2.5 ± 0.3	0	33.5 ± 6.4	10.9 ± 1.8	0	28.9 ± 4.5	24.3 ± 1.0	(n = 3)
Indomethacin 50 µmol/l	0	0	0	0	0	73.3 ± 2.7	26.4 ± 2.8	(n = 3)

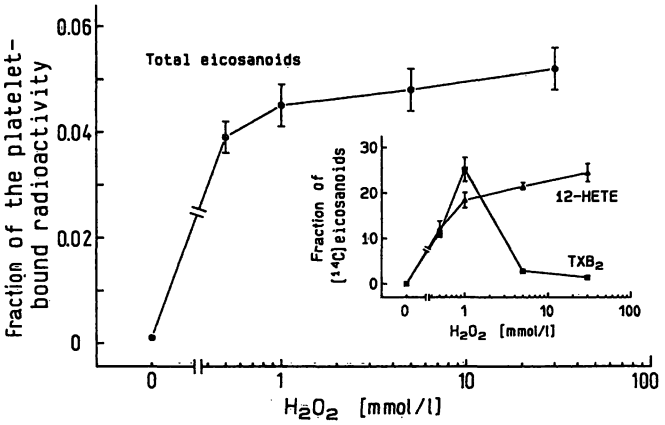

Fig. 2. Effect of hydrogen peroxide (0.5–30 mmol/l) on the liberation of [^{14}C]eicosanoids from platelets prelabelled with [^{14}C]arachidonate. The incubation time was 1 minute. The formation of [^{14}C]eicosanoids is given as fraction of the platelet-bound radioactivity. Inset: Formation of [^{14}C]TXB_2 and [^{14}C]12-HETE as fraction of the total [^{14}C]eicosanoids released. n = 3.
TXB_2 = thromboxane B_2
12-HETE = 12(S)-hydroxy-5,8,10,14-eicosatetraenic acid.

Tab. 2. Effect of iloprost and LU 41.453 on the [^{14}C]eicosanoid release induced by methyl mercury and thrombin. The [^{14}C]arachidonate-prelabelled platelets were stimulated for 2 min. Liberated radioactivity is given as the percentage of the platelet-bound radioactivity.

	Release of [^{14}C]eicosanoids (%)	
	Methyl mercury 20 µmol/l (n = 3)	Thrombin 120 IU/l (n = 3)
Control	5.3 ± 0.5	4.0 ± 0.3
Iloprost 50 nmol/l	4.6 ± 0.3	0.21 ± 0.03
LU 41.453 1 µmol/l	n. s.	$p < 0.01$

mercury strongly depended on the platelet concentration. Equipotent concentrations were 3 µmol/l with $200 \cdot 10^9$/l and 20 µmol/l with $2 \cdot 10^{12}$/l platelets, as used in the experiments on platelet function and metabolism, respectively.

In the presence of indomethacin and in platelets pretreated with acetylsalicylic acid, both H_2O_2 (≤ 2 mmol/l) and methyl mercury (≤ 3 µmol/l) produced neither shape change nor aggregation and [^{3}H]serotonin release (tab. 3), whereas they still liberated eicosanoids (tab. 1). The same results were obtained with the TXA_2/PGH_2-receptor antagonist, sulotroban, and the functional antagonists, iloprost plus LU 41.453 (tab. 2 and 3). In contrast, the thromboxane synthase inhibitor, dazoxiben, augmented the platelet responses to H_2O_2 and methyl mercury (tab. 3).

In order to detect a cytolytic effect of H_2O_2 and methyl mercury, lactate dehydrogenase was measured in the platelet supernatants after 5 min incubation of $200 \cdot 10^9$/l or $2 \cdot 10^{12}$/l platelets with various concentrations of those agents. Even with the highest concentrations tested (30 mmol/l for H_2O_2 and 0.1 mmol/l for methyl mercury), free lactate dehydrogenase did not exceed 3% with H_2O_2 and 4% with methyl mercury of the total platelet lactate dehydrogenase activity. In control experiments neither H_2O_2 (1 and 10 mmol/l) nor methyl mercury (0.01 and 0.1 mmol/l) inhibited the lactate dehydrogenase in the platelet lysate.

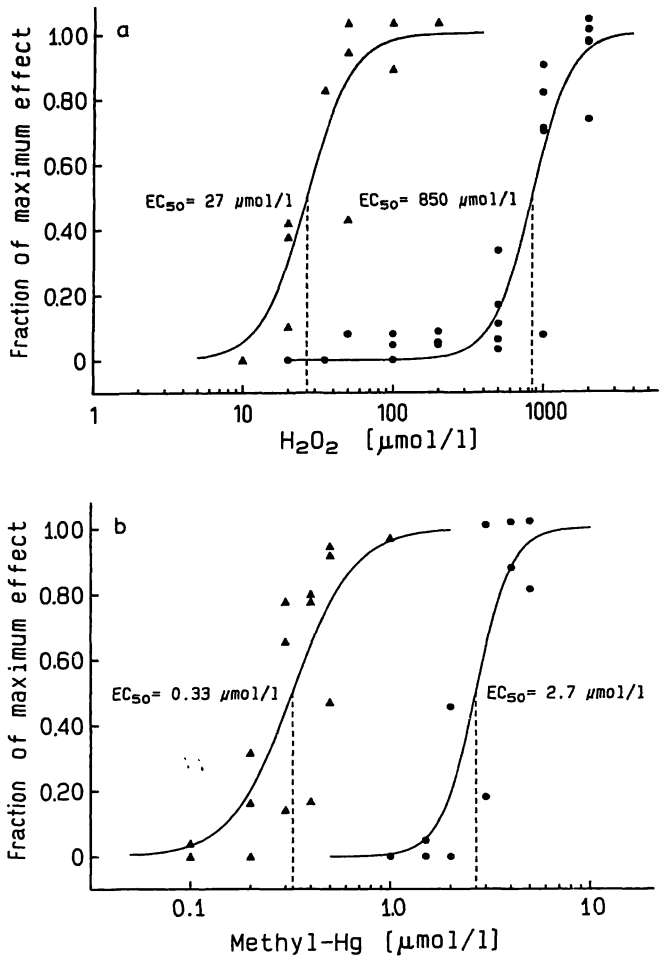

Fig. 3. Concentration effect curves for the shape change (▲—▲) and the aggregation (●—●) induced by (a) hydrogen peroxide and (b) methyl mercury (methyl-Hg). The symbols represent the means of 3—4 experiments in 3 independent experimental series. A sigmoidal, three parameter logistic function was calculated from the data by multiple iterations with a computer program. The bottom value was set constant to 0, and the top value, log(EC_{50}) and the slope were estimated after multiple iterations. The original values for the effects were then normalized by means of the estimated top value (= 1.00).

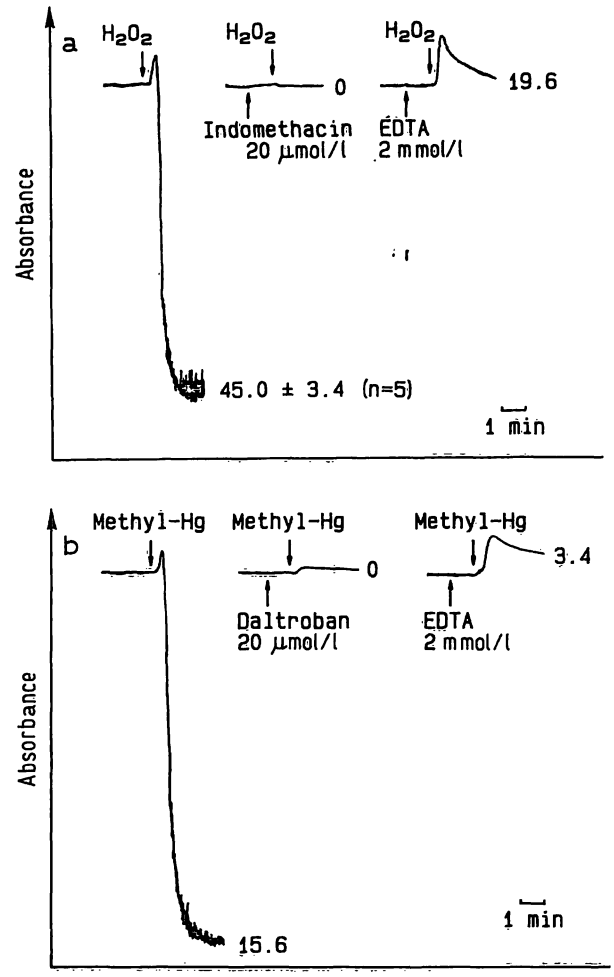

Fig. 4. Effects of indomethacin, daltroban and EDTA on platelet shape change, aggregation and [^{3}H]serotonin release induced by (a) hydrogen peroxide and (b) methyl mercury (methyl-Hg). The platelet-suspending medium contained fibrinogen 300 mg/l.
a: Platelet activation induced by H_2O_2 (2 mmol/l);
left side: control;
middle: indomethacin prevents any platelet response;
right side: EDTA suppresses aggregation and reduces [^{3}H]serotonin release, while a full shape change remains unaffected.
b: Platelet activation induced by methyl mercury (20 µmol/l);
left side: control;
middle: the thromboxane receptor antagonist daltroban prevents any platelet response;
right side: EDTA abolishes aggregation and diminishes [^{3}H]serotonin release without affecting the shape change. The numbers at the ends of the curves indicate the percentage of [^{3}H]serotonin release.

Tab. 3. Effect of indomethacin, dazoxiben and solutroban on shape change, aggregation and [^{3}H]serotonin release induced by various concentrations of H_2O_2 and methyl mercury (methyl-Hg). [^{3}H]Serotonin release was determined upon stimulation in the presence of EDTA 2 mmol/l to avoid aggregation. n = 5.

	Shape change (%)		Aggregation (%)		[^{3}H]Serotonin release (%)
	H_2O_2 500 µmol/l	Methyl-Hg 0.5 µmol/l	H_2O_2 1 mmol/l	Methyl-Hg 5 µmol/l	H_2O_2 2 mmol/l
Control	69.6 ± 3.3	69.5 ± 9.2	68.3 ± 1.0	63.6 ± 2.3	22.1 ± 1.6
Indomethacin 20 µmol/l	0	0	0	0	< 1
Dazoxiben 20 µmol/l	99.7 ± 0.6	97.0 ± 4.8	79.9 ± 4.1	79.8 ± 5.6	56.5 ± 3.2
Sulotroban 20 µmol/l	0	0	0	0	< 1

Discussion

H_2O_2 and methyl mercury share with thrombin the ability to induce a platelet activation accompanied by the formation of the platelet-derived eicosanoids arachidonate, TXB_2, HHT and 12-HETE. They appear in the same concentration ratios with either stimulus, showing that the metabolism of free arachidonate follows its normal routes via 12-lipoxygenase[2]) to 12-HETE and via cyclooxygenase[2]) and thromboxane synthase[2]) to PGH_2 and TXA_2; the stable but inactive TXB_2 and HHT arise from the latter two compounds (41, 42). The unstable intermediary products, PGH_2 and TXA_2, cause shape change, aggregation and secretion, which can be suppressed by inhibitors of their formation such as acetylsalicylic acid and indomethacin, or by inhibitors of their action such as the PGH_2/TXA_2 receptor antagonists, sulotroban (43) and daltroban (44). The thromboxane synthase inhibitor, dazoxiben, enhances the platelet response (29) due to accumulated PGH_2, which shares the same receptor as TXA_2 (45). Acetylsalicylic acid (46) or daltroban reduce but never suppress the platelet responses to thrombin, ADP, platelet activating factor, serotonin or the stable thromboxane mimetic U 46619 (1). In contrast, acetylsalicylic acid, sulotroban and daltroban prevented the platelet stimulation by H_2O_2 and methyl mercury. Therefore, platelet stimulation by H_2O_2 and methyl mercury essentially requires the formation and action of PGH_2 and TXA_2, whereas each of the other mentioned agonists also elicits a platelet response independently of PGH_2 and TXA_2 formation. This implicates different mechanisms for triggering eicosanoid formation with H_2O_2 or methyl mercury and with the other physiological agonists.

Thrombin, ADP, platelet activating factor, serotonin and U 46619 act on platelets via specific receptors. Their platelet-activating signal is transmitted by an activation of the phospholipase C and the formation of inositol-1,4,5-trisphosphate and diacylglycerol, which increase the intracellular free Ca^{2+} concentration and activate protein kinase C, respectively (47, 48). These events lead to shape change, aggregation and secretion and can be inhibited by agents that stimulate the cyclic AMP formation, such as prostacyclin (49, 50). Therefore, agents capable of stimulating cAMP accumulation can be expected to inhibit the thrombin-induced eicosanoid release, if this release depends on the thrombin-induced activation cascade. In fact, the stable prostacyclin mimetic, iloprost (51), and the cyclic AMP phosphodiesterase inhibitor, LU 41.453, inhibited the thrombin-induced eicosanoid release, but did not reduce the mobilization of arachidonate and the formation of eicosanoids caused by methyl mercury. This confirms that eicosanoid release is secondary to platelet activation by thrombin, whereas methyl mercury does not require the activation cascade for its effect on eicosanoid release. The primary effect of H_2O_2 and methyl mercury on arachidonate mobilization is also demonstrated by the observation that indomethacin does not suppress the eicosanoid release, although it prevents the H_2O_2- and methyl mercury-induced shape change, aggregation and serotonin secretion.

A potent stimulus for eicosanoid release in platelets is an intimate platelet contact (5), an effect which is most pronounced in citrated plasma (52). Therefore, if platelets are allowed to aggregate, their aggregation and secretion response is enhanced as a consequence of PGH_2/TXA_2 formation. The aggregation (= contact) can be definitely suppressed by EDTA, which interferes with the binding of the aggregation cofactor, fibrinogen, to its glycoprotein IIb/IIIa receptors on the platelet surface (53). Even in the presence of EDTA, H_2O_2 2 mmol/l and methyl mercury 3 µmol/l induced a full shape change and a certain release of serotonin. Thus, these agents were potent stimuli of PGH_2/TXA_2 formation, even in the absence of an aggregation-induced secondary mobilization of arachidonate.

Higher concentrations than 2 mmol/l of H_2O_2 and 3 µmol/l of methyl mercury were not used for specific stimulations of the eicosanoid release, because of certain side effects. H_2O_2 (5 – 30 mmol/l) still enhanced the eicosanoid release and 12-HETE production, but suppressed the formation of TXB_2 and HHT. This is consistent with the inhibition of the cyclooxygenase observed by *Kawaguchi* et al. (24) in rabbit platelets. In addition, oxygen bubbles were formed when H_2O_2 was present at $\geq$ 1.5 mmol/l, due to the instability of H_2O_2 at high concentrations. When more than 3 or 20 µmol/l of methyl mercury were applied to 200 $\cdot 10^9$/l or 2 $\cdot 10^{12}$/l platelets, respectively, shape change and aggregation occurred, despite the presence of indomethacin. In other words, methyl mercury at these high concentrations elicits another platelet-activating effect that is not mediated by the formation and action of PGH_2 and TXA_2. Even at the highest concentrations tested, lactate dehydrogenase liberation remained below 3% with 30 mmol/l H_2O_2 and 4% with 1 mmol/l methyl mercury, showing that cell lysis did not occur to an appreciable degree.

The mechanism whereby H_2O_2 and methyl mercury selectively trigger the release of arachidonate remains to be elucidated. In platelets, free arachidonate is liberated mainly by phospholipase A_2 activated by cytoplasmic Ca^{2+}, which increases during platelet ac-

tivation (54). This mechanism is thought to be operative in the thrombin-induced eicosanoid release (55, 56) but is unlikely to be involved in the eicosanoid release induced by H_2O_2 or methyl mercury. A small rise in cytoplasmic Ca^{2+} is sensitively reflected by a spherization of the platelets (57). In platelets exposed to acetylsalicylic acid however, no shape change is observed upon stimulation with H_2O_2 or methyl mercury, despite considerable arachidonate release. Whether H_2O_2 or methyl mercury exert a direct, Ca^{2+}-independent effect on the phospholipase A_2[2]) activity or interfere with the reacylating pathway of arachidonate is currently under investigation in our laboratory. Both the phospholipase A_2 (58) and the reacylating enzymes, arachidonyl-CoA synthetase[2]) (16) and lysophospholipid acyltransferase[2]) (17), have high activities in platelet membranes, which suggests a high turnover rate of arachidonate deacylation and reacylation. Under these conditions, an inhibition of the reacylating enzymes could just as well lead to a rise in free arachidonate, as to an activation of the phospholipase A_2. Since in contrast to thrombin and other physiological agonists, H_2O_2 and methyl mercury have a direct effect on arachidonate release, these agents are valuable tools for investigating the biochemical mechanism of arachidonate mobilization. Irrespective of whether their target(s) is (are) located within the phospholipase A_2 or the reacylation pathway, they provide models which not only permit the study of the regulation of free arachidonate in platelets, but also provide an experimental approach to its specific inhibition. Specific drugs that suppresss arachidonate mobilization by an inhibitory effect on phospholipase A_2 or a stimulating effect on the reacylating enzymes are not yet available. The use of specific stimuli of arachidonate release, such as H_2O_2 and methyl mercury, should facilitate the search for and the characterization of selective inhibitors of arachidonate release.

Acknowledgement

The authors thank *Rita Wipplinger* and *Ute Thömen* for their expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft, Pa 263.

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